REMARKS

The specification has been amended on the lines of the amendments presented in the parent application. In addition, the status of this application has been set forth. The present application is directed to the subject matter of original claims 12 and 13. Claims 1-11 are cancelled without prejudice.

The Patent Office is requested to use the computer-readable version of the Sequence Listing of the parent application in connection with the present application. A paper copy of the Sequence Listing presented in the parent application is presented herewith.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached pages are captioned "**Version with markings to show changes made**".

Favorable action on the merits is solicited.

Respectfully submitted,

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A method for analysing a polynucleotide containing a variable sequence and a set or array of oligonucleotides therefor

~~DETECTING DNA SEQUENCE VARIATIONS~~

This application is a divisional of ~~Serial No. 09/502,778 filed February 11, 2000~~, which is a divisional of ~~Serial No. 08/930,798~~ filed October 6, 1997.

~~INTRODUCTION~~ BACKGROUND OF THE INVENTION

Detection of variation in DNA sequences forms the basis of many applications in modern genetic analysis: it is used in linkage analysis to track disease genes in human pedigrees or economically important traits in animal and plant breeding programmes; it forms the basis of fingerprinting methods used in forensic and paternity testing [Krawczak and Schmidtke, 1994]; it is used to discover mutations in biologically and clinically important genes [Cooper and Krawczak, 1989]. The importance of DNA polymorphism is underlined by the large number of methods that have been developed to detect and measure it [Cotton, 1993]. Most of these methods depend on one of two analytical procedures, gel electrophoresis or molecular reassociation, to detect sequence variation. Each of these powerful procedures has its drawbacks. Gel electrophoresis has very high resolving power, and is especially useful for the detection of variation in the mini- and microsatellite markers that are used in linkage analysis and fingerprinting; it is also the method used to analyse the variation found in the triplet repeats that cause a number of mutations now known to be the cause of around ten genetic disorders in humans [Willems, 1994]. Despite its great success and widespread use, gel electrophoresis has proved difficult to automate: even the systems which automate data collection require manual gel preparation; and as samples are loaded by hand, it is easy to confuse samples. The continuous reading electrophoresis machines are expensive, and manual analysis is technically demanding, so that its use is confined to specialised laboratories which have a high throughput. Furthermore, difficulties in measuring fragment size preclude rigorous statistical analysis of the

results.

By contrast, oligonucleotide hybridisation lends itself to automation and to quantitative analysis [Southern *et al.*, 1992], but it is not well suited to the analysis of variation in the number of repeats in the micro- and minisatellites, as the small fractional change in the number of repeats produces a barely detectable change in signal strength; and of course it would not be possible to distinguish two alleles in the same sample as each would contribute to a single intensity measurement. Thus, many different combinations of alleles would produce the same signal. Present hybridisation methods are much better suited to analysing variation in the DNA due to point mutation - base substitution deletions and insertions, for which it is possible to design allele specific oligonucleotides (ASOs) that recognise both the wild type and the mutant sequences [Conner *et al.*, 1983]. Thus it is possible in principle, in a relatively simple test, to detect all possible genotypes. However, a problem that arises in practice in the use of oligonucleotide hybridisation is that in some cases the extent of reassociation is barely affected by a mismatched base pair.

~~THE INVENTION~~ BRIEF SUMMARY OF THE INVENTION

The invention describes a general approach which can be applied to all forms of variation commonly used as DNA markers for genetic analysis. It combines sequence-specific hybridisation to oligonucleotides, which in the preferred embodiment are tethered to a solid support, with enzymatic reactions which enhance the discrimination between matching and non-matching duplexes, and at the same time provide a way of attaching a label to indicate when or which reaction has taken place. Two enzymatic reactions, chain extension by DNA dependent DNA polymerases and DNA strand-joining by DNA ligases, are dependent on perfect matching of sequences at or around the point of extension or joining. As we shall show, there are several ways in which these enzymes

oligonucleotide with the second intermediate oligonucleotide; and repeating the steps with oligonucleotides tethered to different locations of the support

BRIEF DESCRIPTION OF THE DRAWINGS

Reference is directed to the accompanying drawings in which each of Figures 1 to 6 is a series of diagrams illustrating a method according to the invention.

Figure 1 shows detection of point mutation by single base extension.

Figure 2 shows detection of point mutation by hybridisation to allele specific oligonucleotides and chain extension.

Figure 3A shows detection of point mutation by tag ligation to allele specific oligonucleotides.

Figure 3B shows detection of point mutation by ligation to library of differentially tagged allele specific oligonucleotides.

Figure 4A shows analysis of variable numbered tandem repeats by ligation of tag to allelic variants.

Figure 4B shows analysis of variable number tandem repeats by ligation of tag to allelic variants.

Figure 5 shows measurement of variable number tandem repeats by labelled chain extension.

Figure 6 shows analysis of variable number tandem repeats by ligation of tag followed by chain extension.

DETAILED DESCRIPTION OF THE INVENTION

Detection of point mutation

I. Single base-specific extension of tethered primers.

In this application, the tethered oligonucleotide terminates at a position one base before the variable base in the target sequence (Fig.1). A nucleotide precursor triphosphate or dideoxyribonucleotide triphosphate, labelled, for example with a fluorescent tag, is added in the

DNA polymerase

The M13 sequencing primer - 5'-GTAAAACGACGGCCAGT-3' (SEQ ID NO. 1) - attached to aminated polypropylene through its 5' end was synthesised as described. A solution of M13 DNA (single-strand, replicative form, 0.1 µl, 200 ng/µl) was applied in two small spots to the surface of the derivatised polypropylene. A solution containing three non-radioactive deoxyribonucleotide triphosphates, dATP, dGTP, TTP (10 µmol each), α^{32}P-dCTP (10 µCi), Taq DNA polymerase and appropriate salts, was applied over a large area of the polypropylene, including the area where the M13 DNA had been spotted. The polypropylene was incubated at 37°C for 1 hr in a vapour saturated chamber. It was then washed in 1% SDS at 100°C for one minute, and exposed to a storage phosphor screen for one minute and scanned in a phosphorimager. The regions where the DNA had been applied showed a high level of radioactivity, against a low background where no DNA had been applied. This experiment shows that oligonucleotides tethered to a solid support can act as primers for DNA-dependent synthesis by DNA polymerase, as required for applications using this enzyme for mutation detection.

Experiments described below show that both polynucleotide kinase and DNA ligase can be used to modify oligonucleotides tethered to a solid support. There are several ways in which phosphorylated oligonucleotides and the ligase reaction can be used to detect sequence variation.

Methods for Making Arrays of Sequence Variants.

1. Allele specific oligonucleotides for point mutations.

For the preferred embodiment, it will be necessary to use oligonucleotides tethered to a solid support. The support may take the form of particles, for example, glass spheres, or magnetic beads. In this

more nucleotides. The short repeats may be made using chemical synthesis; in the case of inserts with large numbers of repeat units, it would be more economical to use a synthetic route which used the repeat unit as a reactant, rather than building them up one base at a time; such methods have been used to make polynucleotides by chemical synthesis. An attractive alternative would be to build the repeat units by ligating the monomer units; they could be added stepwise, one unit at a time provided a method could be found to block one end to prevent polymerisation; for example the oligonucleotide building block may be terminated by a hydroxyl group, which is then phosphorylated after ligation so that the unit becomes an acceptor for the next one; the monomer may have a phosphate group protected by a cleavable group, such as a photocleavable group, which can be removed after ligation to allow a subsequent ligation [Pillai, 1980]. A second alternative, which would be especially favourable for longer units such the minisatellites, would be to attach either cloned or enzymatically amplified molecules to the solid support. For example, each variant sequence could be amplified by the PCR, using a biotinylated oligonucleotide for one of the primers. The strand starting with this group could then be attached to a streptavidin coated surface, and the other strand removed by melting [Stahl *et al.*, 1988].

EXAMPLE 1

DEMONSTRATION OF THE ANALYSIS OF LENGTH POLYMORPHISM BY LIGATION TO AN ARRAY OF VNTRs

An array of VNTRs was made as described in Fig. 4b, in which the anchor sequence was 5'-tgtagtggtgtgatcaaggc-3' (SEQ ID NO. 2). The repeat unit was 5'-cttt-3'; stripes, ca 3 mm wide, of sequence variants of the form: Anchor-Repeat$_N$, with N = 4-10, were made as stripes on the surface of a

sheet of polypropylene. The synthesis was carried out using 3'-deoxyribophosphoramidites, this chemical orientation produces oligonucleotides tethered through their 3' ends to the polypropylene, and a free 5' hydroxyl group. To create a substrate for ligation, this OH group was phosphorylated by immersing a strip of the polypropylene (3mm x 18mm), carrying the array of oligonucleotides, in 0.5 ml of a solution containing 4mM ATP and 77.6 units of polynucleotide kinase with buffer and Mg^{++} according to the supplier's instructions. The reaction was left for 6 hours at 37°C; the strip was removed and immersed in boiling water to kill the polynucleotide kinase. The target sequences, which are complementary to elements of the array oligonucleotides and to the ligation tag, 5'-Anchor-$Repeat_{10}$-Tag and 5'-Anchor-$Repeat_5$-Tag were added to 0.5 ml of a solution, preheated to 95°C, containing the tag, 5'-gtggtcactaaagtttctgct-3' (SEQ ID NO:3), which had been labelled at its 5' end using polynucleotide kinase and ^{33}P-gamma-ATP, thermal ligase (500 units), and buffer and salts according to the supplier's instructions. The polypropylene strip was immersed in the hot solution, which was then allowed to cool to 68°C, and left at this temperature for 16 hours. The polypropylene strip was removed and placed in 25% formamide at 95°C for 5 minutes, rinsed in water at the same temperature, dried and exposed to a storage phosphor screen, from which an image of the radioactivity was collected. The results showed counts close to background over most of the array; counts on Anchor-$Repeat_5$ and Anchor-$Repeat_{10}$ were more than five times those over adjacent cells in the array. This experiment indicates that the ligase is able to distinguish length variants of the repeat sequence and gives optimum ligation only when the number of repeats in the target matches that in the allele specific oligonucleotide in the array. Thus, it should easily be possible to detect the two allelic variants in a heterozygote.

Ligation and/or polymerisation is possible if the

oligonucleotide is tethered through the 5' end . The oligonucleotides can be synthesised *in situ* using deoxyribophosphoramidites with 3' dimethoxytrityl groups and 5' phosphoramidite (reverse phosphoramidites). It is unnecessary to phosphorylate the tethered polynucleotide for ligation assays since the phosphate needed for ligation is provided by the tag oligonucleotide.

EXAMPLE 2

Analysis of VNTR lengths

An array of VNTR's was made as described in Figure 4B, with the oligonucleotides anchored through their 5' ends. The repeat unit was 5' ttca and the anchoring sequence 5' cttatttccctca (SEQ ID NO.4). Stripes 6mm wide of sequence variants of the form: Anchor-Repeat$_N$ where N= 4-8 were made on the surface of a sheet of polypropylene using "reverse" phosphoramidite monomers.

Analysis by ligation

A strip of the array (30mm x 2mm) was immersed in a solution of 600pmols of the target oligonucleotide 5' cacagactccatgg(tgaa)$_6$tgagggaaataag (SEQ ID NO.5), 1.4 pmol of oligo 5' ccatggagtctgtg (SEQ ID NO.6) (labelled at its 5' end using polynucleotide kinase and ^{33}P gamma ATP) and buffer and salts according to the suppliers instructions, the total volume being 293 µl. The solution was heated to 65°C and 7 µl of Tth DNA ligase added. The reaction was then cooled to 37°C and left at that temperature for 18hrs. After removal from the reaction solution the strip was washed in T.E. buffer, blotted dry and exposed to a storage phosphor screen from which an image of the radioactivity was taken. The results showed that the target sequence ligated to the correct sequence with a higher yield than to the shorter and longer sequences in adjacent cells of the array.

first base in the flanking sequence which is absent from the repeat.

A strip of the array from Example 2 (30mm x 2mm) was added to a solution of 500 pmols of the target oligonucleotide 5' cacagactccatgg(tgaa)$_6$tgagggaaataag in buffer and salts at a concentration 1.09 times the suppliers instructions, the total volume being 275 µl. The solution was heated to 75°C for 5 minutes and cooled to 37°C over a period of 25mins. The solution was removed and added to 3.3 pmols ^{32}P alpha dCTP, 5 µl 1M DTT. 13 units of Sequenase version 2.0 and water to give a final volume of 295 µl. This solution was added to the array and heated at 37°C for 15hrs 40mins. The polypropylene strip was removed, washed in water and exposed to a storage phosphor screen. The results showed counts of 5 times more for the correct sequence than shorter sequences and twice for the correct sequence compared with longer repeats.

A similar strip of the array (30mm x 2mm) was added to a solution of 500 pmols of the target oligonucleotide 5' cacagactccatgg(tgaa)$_6$tgagggaaataag in buffer and salts 1.09 times the suppliers instructions, the total volume being 275 µl. The solution was heated to 75°C for 5 mins and cooled to 37°C over a period of 25 mins. The solution was removed and added to 3.3 pmols ^{32}P alpha dTTP, 5 µl 1M DTT, 13 units of Sequenase version 2.0 and water to give a final volume of 295 µl. This solution was added to the array and heated at 37°C for 15hrs 40mins. The polypropylene strip was removed, washed in water and exposed to a storage phosphor screen. The results showed counts of 4.5 times more for the shorter array sequences than the correct and longer repeat lengths.

EXAMPLE 5

VNTR analysis by ligation

In an experiment similar to the one described in Example 1, an array was created using the human fes/fps locus sequence as a target.

The anchor sequence 5' agagatgtagtctcattctttcgccaggctgg 3' (SEQ ID NO. 7) was the actual flanking sequence to the attt repeats of the fes/fps microsatellite (EMBL Accession No X06292 M14209 M14589) as it occurs in human genomic DNA. Using a target oligonucleotide representing the 10 repeat allele and ligating a ^{33}P labelled 5' flanking sequence (5' g gag aca agg ata gca gtt c 3') (SEQ ID NO. 8) and doing a similar experiment to that described above, the resulting radioactivity on the anchor-repeat$_{10}$ cell was over 10 fold that on adjacent cells in the array.

EXAMPLE 6

Demonstration of stepwise ligation of oligonucleotides bound to a solid support.

A primer oligodeoxynucleotide - 5' PO_4 gta aaa cga cgg cca gt 3' (SEQ ID NO. 9) attached to aminated polypropylene through its 3' end, was synthesised and phosphorylated as described. A small square (2mm x 2mm) piece of this material was placed in standard ligation buffer, with template oligonucleotide 5'tcg ttt tac cgt cat gcg tcc tct ctc 3' (SEQ ID NO. 10) (250 nM) and a protected ligator oligonucleotide 5' NB PO_4 cgc atg acg 3' (250 nM) and ^{33}P labelled extender oligonucleotide 5' gag aga gga 3', where NB is a protecting group based on a photocleavable o-nitrobenzyl derivative. The NB protected phosphate of the ligator oligonucleotide had previously been shown to be unable to take part in the ligation reaction. The NB group had also been shown to be removable by uv light to leave a fully functional phosphate group. To this mixture was added thermus thermophilus DNA ligase (Advanced Biotechnologies) 25u and the reaction incubated at room temperature for 6 hours. The mixture was then irradiated with uv light (20 minutes room temperature) and incubated for a further 12 hours. The polypropylene patch was then washed with 30% formamide at 95°C for 5 minutes, and exposed to a storage phosphor screen for 24 hours and scanned in a phosphorimager. The patch showed

a level of radioactivity 50 fold higher than a patch treated in a similar fashion but without addition of the central "ligator" oligonucleotide. In a similar experiment using a phosphorylated ligator oligonucleotide a similar amount of radioactive extender oligonucleotide became covalently attached to a third polypropylene/oligonucleotide primer square.

EXAMPLE 7

Demonstration of point mutation analysis by ligation to allele specific oligonucleotides attached to solid supports

Four tethered ASOs 5' (gca or t)ag aga gga 3', differing only at their 5' base, were synthesised as described above, with the 3' end attached to aminated polypropylene. Phosphorylation was carried out as described and four squares of polypropylene carrying each ASO were placed in standard ligation buffer along with complementary target oligonucleotide 5' tcc tct ctc cgt cat gcg tat cgt tca at 3' (SEQ ID No. 11) (250 nM). After addition of ^{33}P labelled ligator oligonucleotide 5' cgc atg acg 3' (10 nM) and thermus thermophilus DNA ligase (100u), the mixture was incubated at 37°C for 18 hours. The ASO which was fully complementary to the target oligonucleotide was found to have acquired 100-fold greater radioactivity through ligation of the labelled ligator than the non-complementary ASOs.

EXAMPLE 8

Demonstration of DNA ligation specificity

In a model experiment to assess the specificity of TTh DNA ligase, ligator and extender deoxyoligonucleotides were ligated together by means of hybridisation to an oligonucleotide template and ligation by DNA ligase.

Template oligonucleotide 5' tcc tct ctc cgt cat gcg tat cgt tca (SEQ ID No. 12) at 3' (250 nM), phosphorylated, ^{33}P labelled, extender oligonucleotide

5' PO_4gag aga gga 3' (10 nM) and ligator sequence 5' gca gta cg 3' (250 nM) were mixed together in standard ligation buffer with DNA ligase 25 u. This mixture was incubated at 35°C. Samples of this mixture were removed and the reaction stopped by addition of formamide at 15, 30, 60, 120, and 240 minutes. The ligated and unligated products were separated by 20% denaturing polyacrylamide gel electrophoresis. The gel was exposed to a phosphor screen for 18hours and scanned by a phosphorimager. The relative proportions of ligated to unligated products of the reaction were then measured. 50% of the extender sequence had been ligated to the ligator sequence in 30 minutes. By comparison in a similar experiment with ligator 5' gca tga ag 3' after 30 minutes only 1% of the extender sequence had become ligated.

Other polymerases and ligases such as Taq polymerase, Thermosequenase, T4 DNA ligase and *E.Coli* DNA ligase have also been shown to be useful in experiments similar to those described above.

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